Mutations in the Translation Initiation Region of the *pac* Gene Resulting in Increased Levels of Activity of Penicillin G Acylase

Özlem Akkaya[1*], Saliha İşsever Öztürk[1], Albert Bolhuis[2], Füsun Gümüşel[1]

[1]Department of Molecular Biology and Genetics, GYTE / Gebze Institute of Technology, 41400, Kocaeli, Turkey

[2]University of Bath, Department of Pharmacy and Pharmacology, Bath BA2 AY, UK

***Correspondence**

Özlem Akkaya, Department of Molecular Biology and Genetics, Gebze Institute of Technology, 41400, Kocaeli, Turkey.

Phone: +90-262-605 25 42; Fax: +90-262-605 25 05;

E-mail: ozlem@gyte.edu.tr

Running headline: Enhanced expression of *pac*

Abstract Penicillin G acylase (PA) is an important enzyme used in the industrial production of b-lactam antibiotics. In this study, the effects of mutations in the translation initiation region of the *Escherichia coli pac* gene, encoding periplasmic PA, were examined. Several mutations led to increased amounts of PA activity, including those that lengthened the spacer region between the ribosome binding site and the ATG start codon, and those with altered codons on positions +2 and +4 relative to the start codon. These results indicated that the wild-type sequence of the *pac* gene does not provide maximum expression levels and that the strategies applied in this study can be used to improve production of PA in *E. coli*. Unexpectedly, our study also suggested that translocation of PA was, in contrast to earlier reports, shown not to require the Twin-arginine translocation pathway for transport into the periplasm.

Keywords Penicillin G acylase . *pac* gene . translation initiation . codon usage . Tat pathway.

Introduction

Initiation of translation in bacteria is a major determinant of the levels of protein synthesis (Draper 1996). During formation of the translation initiation complex, mRNA and N-formyl-methionyl (fMet)-$tRNA^{fMet}$ bind to ribosomes in a process depending on translation initiation factors (IF1, IF2 and IF3). The molecular interactions present in this translation initiation complex consist of base-pairing of the Shine-Dalgarno (SD) sequences on the mRNA with the anti-SD sequence present in the 16S rRNA gene, together with an interaction between the start codon and the anticodon of fMet-$tRNA^{fMet}$ (Gualerzi et al. 2001).

In order to optimize translation initiation efficiency in bacteria, previous studies mainly focused on four components: (i) the start codon; (ii) the SD-sequence; (iii) the length and composition of the spacer between the start codon and the SD-sequence; and (iv), the upstream and downstream regions flanking the initiation codon (Ma et al. 2002; Shine and Dalgarno 1974; Stenstrom et al. 2001a; 2001b). Among these, the effectiveness of a SD sequence is determined by the base-pairing potential with the anti-SD sequence as well as the spacing from the ATG translational start codon (de Smit and van Duin 1994). The downstream region (4-5 codons) is also important for translation initiation, as codon usage may influence secondary structure and stability of mRNA (de Smit and van Duin 1990; Stenström et al. 2001a). The coding region of the mRNA directly following the start codon has a codon usage that differs from the rest of the gene sequence (Chen and Inouye 1990). Codon changes at +2 can alter gene expression by 15- to 20-fold (Looman et al. 1987; Stenstrom et al. 2001a), and the effect on gene expression can be modulated by subsequent codons as well (Stenstrom et al. 2001b). For instance, the overrepresentation of rare codons such as AGA and AGG among the first 25 codons of genes may alter the efficiency of translation and is important in regulation of protein synthesis (Chen and Inouye 1990).

Penicillin G acylase (PA) is an industrially important enzyme that is used to produce 6-aminopenicillanic acid (6-APA), the starting compound for the synthesis of semisynthetic penicillins (Elander et al. 2003). The precursor is synthesized as an inactive 96-kDa pre-pro-PA, which contains a signal peptide at its N-terminus, mediating translocation into the periplasm through the Twin-arginine translocation (Tat) pathway (Ignatova et al. 2002). Translocated pro-PA is further processed into A (23 kDa) and B (63 kDa) chains by autoproteolytic reactions (Hewitt et al. 2000). Interestingly, the *E. coli pac* gene expression is temperature-sensitive and PA can be only synthesized at growth temperatures below 30ºC (Keilmann et al. 1993).

The canonical Shine-Dalgarno sequence is AAGGAGG, while the sequence upstream of the *pac* gene is GAGGA. This sequence has thus two potential ribosome binding sites (RBS), being either GAGG (SD1) or AGGA (SD2), each of which is able to pair differently to the 3' end of the 16S rRNA (Keilmann et al. 1993; Table 2). The spacing between the two potential SD sequences and the ATG initiation codon (4 or 5 nucleotides) is also shorter than the more typical 7 to 12 nucleotide spacing for *E. coli* genes (Ma et al. 2002). Here we analyzed the production of PA with a number of mutations in the translation initiation region of the *pac* gene. These included altering the spacer region between the RBS and the ATG initiation codon with a 6-8 nucleotide spacer, removal of SD2, and mutations in the region just downstream of the ATG start codon. Interestingly, several of these mutations led to increased production of PA.

Materials and methods

Chemicals

Restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, isopropyl-β-D-thio-galactopyranoside (IPTG) and ampicillin were purchased from Roche or MBI Fermentas. Chloramphenicol was from Sigma-Aldrich. The oligonucleotides used were synthesized by Promega. The constructed plasmids were extracted using the Wizard *Plus* SV Miniprep DNA purification kit of Promega. The presence of the mutations was confirmed by sequencing, using a CEQTM 8000 Genetic Analytic System (Beckman Coulter).

Bacterial strains and plasmids

Escherichia coli strain JM109 (F′ *traD36 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^{-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17*) was used for primary cloning and expression. The plasmid vectors pUC19 (Yanisch-Perron et al. 1985) and pMMB208 (Morales et al. 1991) were used for cloning. While the former carries the *lac* promoter, the latter carries the *tac* promoter. The *E. coli* strains MC4100 (F^{-} *araD139* Δ*lacU169 relA1 rpsL150 thi mot flb5301 deoC7 ptsF25 rbsR*) and DADE (MC4100Δ*tatABCDE*; Wexler et al. 2000) were also the host strains for pMMB208 constructs. For expression studies, strains were cultured at 26ºC in casein medium (0.4% (w/v) casein, 0.8% (w/v) yeast extract, 0.42% (w/v) K_2HPO_4, 0.3% (w/v) KH_2PO_4) supplemented with ampicillin (100 μg/ml) or chloramphenicol (20 μg/ml) when appropriate.

Plasmid constructs

Plasmid *pac*9.5-pEMBL9^{+} (Gümüşel et al. 1996) was used as the template to introduce mutations into the translation initiation region of the *pac* gene and the primers used in this study are shown in Table 1. Firstly, a fragment containing the 2.6-kb *pac* gene was PCR-

amplified from the plasmid *pac*9.5-pEMBL9$^+$ using the primer pair of PA11 and PA12. Following amplification by *Taq* polymerase, the fragments were digested with *Xba*I-*Kpn*I and ligated into pUC19 to generate pUCpac2.6. To introduce the mutations shown in Table 2, a fragment containing a 550-bp region was amplified from *pac*9.5-pEMBL9$^+$ with primer pairs PA6/PA10 (Mut4), PA7/PA10 (Mut5), PA8/PA10 (Mut6) and PA9/PA10 (Mut7). These fragments were digested with *Eco*NI and *Nco*I and ligated into pUCpac2.6, creating pUCpac2.6OA4, pUCpac2.6OA5, pUCpac2.6OA6, and pUCpac2.6OA7 respectively. A 0.9 kb fragment downstream of the *pac* gene, which is required for good expression (Gümüşel et al. 1996; 2001), was amplified from *pac*9.5-pEMBL9$^+$ using primers PA14 and PA16. The resulting PCR product was cloned into the *Kpn*I and *Eco*NI sites of pUC19 to generate pUC19-900. Next, the 2.6 kb *Xba*I-*Kpn*I inserts from the pUCpac2.6OA plasmids were cloned into pUC19-900, resulting in the final constructs, which were named pUC-PACOA4, pUC-PACOA5, pUC-PACOA6 and pUC-PACOA7, respectively.

For expression in a low-copy number vector, *Eco*RI/*Hind*III fragments were re-cloned into pMMB208 (Morales et al. 1991) under the control of a *tac* promoter. These constructs were denoted pMMB-PACwt, pMMB-PACOA6 and pMMB-PACOA7. All pUC and pMMB constructs were verified by sequencing.

Expression and Activity

To evaluate the enzymatic activity of PA, *E. coli* JM109 cells carrying the plasmids described above were grown in casein medium at 26ºC or 37ºC for 20 h. When the OD_{600} reached 0.6-0.8, 0.1 mM Isopropyl-β-D-thio-galactoside (IPTG) was added to induce expression. PA enzyme activity was measured as described by Balasingham et al. (1972). Standard assay conditions for enzyme activity assays were as follows: 0.0125 ml enzyme

solution was added to 0.488 ml of 15 mM penicillin G in 50 mM phosphate buffer at pH 8.0 and the mixture was incubated at 40ºC for 1 h. Activity was determined by measuring the absorption at 415 nm, and cell density was determined by measuring the optical density at 600 nm (OD_{600}). One unit is defined as the amount of enzyme required to produce one µmol of 6-APA per min, and activity was expressed as units per OD_{600}.

Western blotting

Proteins were resolved on 12% SDS-PAGE gels prepared according to Laemmli (1970). For blotting, proteins were transferred to nitrocellulose membranes (Sartorius, pore size 0.45 µm) and immunoblotting was performed according to Towbin et al. (1979), using a Mouse-polyclonal antibody against PA as primary antibody. The secondary antibody was alkaline phosphatase-conjugated anti-mouse IgG. Binding was visualized with 5-bromo-4-chloro-3-indoyl phosphate and Nitroblue Tetrazolium.

Results

Construction of mutations in the translation initiation region

Several plasmids containing mutations in the translation initiation region of the *pac* gene were constructed, and these are listed in Table 2. Even though growth rates were shown not to affect PA production in *E. coli* (Chou et al. 1999), we did determine those for all strains constructed. Significant differences in growth rates were however not observed (data not shown). All studies were performed with 0.1 mM IPTG for induction, which provided maximal production levels under the conditions tested (data not shown).

Effects of the SD and spacer region mutations on the expression of the *pac* gene

The first aim was to find out whether mutations in the SD sequence and/or spacer region had any effect on the production of active PA. To achieve these plasmids containing the mutations listed in Table 2 were constructed.

The spacer between the SD1/SD2 ribosome binding site and the ATG start codon is 4/5 nucleotides, which is shorter then normally found (Ma et al. 2002). To investigate the effect of altering this spacer, mutations were created with spacers of 6 (S_{pac}, namely Mut3 in this study) and 8 (Mut5) nucleotides (Table 2). Both mutations led to an increase in active PA production: approximately five-fold with a spacer of 6 nucleotides, and nearly 12-fold with a spacer of 8 nucleotides (Fig.1).

To check the importance of a complete SD2, the second A in the GAGGA sequence upstream of the start codon in Mut3 was changed to a T (Mut4). As a result, production of active PA levels increased 61% (compare Mut3 with Mut4; Fig. 1). This suggests that the presence of an intact SD2 reduces protein synthesis levels. For the all mutations mentioned above, activity was only observed at 26^{o}C and not at 37^{o}C (not shown). This corroborated earlier findings that *pac* gene expression is temperature-sensitive (Keilmann et al. 1993).

Downstream region effects on *pac* gene expression

The first few codons in the coding region may also influence translation initiation, for instance, through changes in the secondary structure of the mRNA. The mutations that were introduced altered the codons at position +2 (K2R; Mut6) or +4 (R4K; Mut7); both mutations had the same spacer sequence as Mut3 (Table 2). The mutations did not alter the charge of the signal peptide, but it should be noted that Arg4 is part of the (atypical) Tat motif that has been

proposed to direct translocation of PA to the periplasm (Ignotava et al. 2002). As shown in Fig. 1, production of active PA with the Mut6 (+2 codon) construct was two-fold that of levels achieved with Mut3, while the change in PA activity was more moderate (~65% increase) when the codon at +4 (Mut7) was altered. In both cases, PA activity was markedly enhanced compared to the activity achieved with the wild-type (pac_{wt}) sequence. Similar to Mut3/4/5, PA activity was only observed at 26°C and not at 37°C.

The Mut6 and Mut7 mutants had altered signal peptides. To verify that translocation was normal, Tat-deficient *E. coli* strain DADE and its parental strain *E. coli* MC4100 were transformed with the pac_{wt} (Gümüşel et al. 2001) and Mut6/7 constructs. However, the pUC19 derivatives carrying the *pac* gene were not stable in these strains, resulting in deletions of (parts of) the *pac* gene or loss of the entire plasmid (data not shown). This instability in *E. coli* MC4100 and DADE was alleviated by using the low-copy number vector pMMB208 (Morales et al. 1991) instead. With these constructs an increase in total PA activity was observed when compared to the activity of the strains carrying the pUC19 variants. This was also seen using *E. coli* JM109 with the pMMB constructs (not shown), indicating that the increased expression levels were due to the vector used and not the *E. coli* strain. Moreover, maximal PA production, corrected for biomass, was reached faster with strains carrying pMMB constructs compared to those carrying pUC constructs (5 and 20 hours after IPTG induction, respectively; data not shown). There was no significant different in activity/OD600 between 5 and 20 hours for the strains carrying pMMB constructs (data not shown). Surprisingly, when we compared pMMB constructs with pac_{wt} and Mut6/7 variants, we observed hardly any differences in PA activity (Fig. 2).

Unexpectedly, in the Tat-deficient strain, PA activity was only around 30% lower than found in the parental strain (Fig. 2), indicating the absence of a Tat-system did not have a major effect on translocation of PA. To verify this, samples were analyzed by Western

blotting with an antibody that recognizes (pre)-pro-PA and the B subunit (but not the A subunit). As shown in Fig. 3, similar levels of the B subunit, which is indicative of translocation and processing of PA, were observed in all strains including in *E. coli* DADE. Thus, under the conditions tested PA does not appear to be translocated in a Tat-dependent manner.

Discussion

In the present study we show that a number of mutations in the translation initiation region of the *E. coli pac* gene led to increased PA activity. Firstly, the length of the spacer between the SD and the start codon is an important determinant, as PA activity increases markedly when the spacer is lengthened from 4 to 6 or 8 nucleotides. This is not unusual as it has been shown that the optimal spacer length between the SD motif and the initiation codon is between 7 to 12 nucleotides (Ma et al. 2002). Note that the mutated spacer sequences used in this study are enriched with A/T nucleotides, which could also have contributed to increased translation initiation (Hwang et al. 1990).

The sequence of the RBS of the *pac* gene is GAGGA, which can interact in two different ways with the 3' end of the 16S rRNA sequence (Keilmann et al. 1993), with SD1 being GAGG and SD2 being AGGA. We investigated this by changing the second A into a T, which resulted in a further increase in PA activity. This suggests that there is a competition between SD1 and SD2, with binding to SD2 resulting in a lower rate of translation. That might be caused by a difference in spacer length if binding to either SD1 or SD2 is a determinant for efficient production. However, an alternative explanation is that SD2 is never used and that it is a change in the composition of the spacer that influences the rate of

translation by, for instance, influencing the secondary structure of the mRNA (Laursen et al. 2002).

In addition to the RBS and spacer region between the AUG and SD sequence, the downstream region has also been shown to be involved in translation enhancement of *E. coli* gene expression (Stenström et al. 2001a; 2001b). Both Mut6 and Mut7 mutations, having altered nucleotides at codons +2 and +4, resulted in increased amounts of PA activity. There may be a number of reasons for this, such as the use of the rare AGA codon (Arg) in the second codon position for Mut6 (Chen and Inouye 1990; Zalucki et al. 2009), and the A-rich downstream region for Mut7 (Brock et al. 2007). However, we only determined the amount of active (thus secreted) PA, and a more detailed analysis will therefore be performed in a future study.

Improvement of the amount of PA activity in the mutants was not observed when the low copy number vector pMMB was used for expression of the *pac* gene. The reason for this is not clear, but it is conceivable that the level of expression achieved through the more efficient *tac* promoter (de Boer et al. 1983) on pMMB vectors could not be further improved through alterations in the translation initiation region of the *pac* gene, while such improvements could be seen with the weaker *lac* promoter on pUC plasmids.

Surprisingly, translocation of PA to the periplasm was not affected significantly in a strain that lacks all *tat* genes, while it had been suggested that PA is a substrate of the Tat pathway (Ignatova et al. 2002). Notably, the Tat motif in the PA signal peptide (RNRMIVN) is quite distinct from the consensus *E. coli* Tat motif S/TRRxFLK (Berks 1996). Only very few natural Tat substrates have been found lacking one of the two arginine residues, an example being the TtrB subunit of *Salmonella enterica* tetrathionate reductase. However, in the case of TtrB it was shown that the single Arg residue in the Tat motif is essential for translocation; even a conservative replacement (Lys) prevented transport (Hinsley et al.

2001). In the case of PA, replacing Arg6 with Lys (Ignatova et al. 2002) or Arg4 with a Lys (this study) did not affect transport. It thus seems more likely that PA does not rely fully (if at all) on the Tat pathway, but mainly uses the most common route for export in *E. coli*, the Sec pathway (Natale et al. 2008). Interestingly, *E. coli* PA is also transported independently of the Tat pathway in *Pseudomonas aeruginosa* (Krzeslak et al. 2009), which corroborates our suggestion that PA is a Sec-dependent protein.

Acknowledgments Regrettably, during preparation of this paper Prof Füsun Gümüşel passed away, and we dedicate this paper to her memory. The authors would like to thank Prof. Michael Bagdasarian for plasmid pMMB208 used in this study. Additionally, we would like to thank our lab members for their assistance. This work was supported by the Research Fund of Gebze Institute of Technology (BAP, 2006-A4).

References

Balasingham K, Warburton D, Dunnill P, Lilly MD (1972) The isolation and kinetics of penicillin amidase from *Escherichia coli*. Biochim Biophys Acta 276:250-6

Berks BC (1996) A common export pathway for proteins binding complex redox cofactors? Mol Microbiol 22:393–404

Brock JE, Paz RL, Cottle P, Janssen GR (2007) Naturally occurring adenines within mRNA coding sequences affect ribosome binding and expression in *Escherichia coli*. J Bacteriol 189:501–510

Chen GF, Inouye M (1990) Suppression of the negative effect of minor arginine codons on gene expression; preferential usage of minor codons within the first 25 codons of the *Escherichia coli* genes. Nucleic Acids Res 18:1465-1473

Chou CP, Kuo B-Y, Lin W-J (1999a) Optimization of the host/vector system and culture conditions for production of penicillin acylase in *Escherichia coli*. J Biosci Bioeng 88:160–167

de Boer AH, Comstock JL, Vasser M (1983) The *tac* promoter: A functional hybrid derived from the *trp* and *lac* promoters. Proc Natl Acad Sci USA 80:21-25

de Smit MH, van Duin J (1990) Control of prokaryotic translational initiation by mRNA secondary structure. Prog Nucleic Acid Res Mol Biol 38:1–35

de Smit MH, van Duin J (1994) Translational initiation on structured messengers. Another role for the Shine-Dalgarno interaction. J Mol Biol 235:173-184

Draper DE (1996) *Escherichia coli and Salmonella, Volume I: Translational Initiation,* 2nd edn. Washington, DC: ASM Press, pp. 902–908

Elander RP (2003) Industrial production of beta-lactam antibiotics. Appl Microbiol Biotechnol 61:385-392

Gualerzi CO, Brandi L, Caserta E, Garofalo C, Lammi M, La Teana A, Petrelli D, Spurio R, Tomšic J, Pon CL (2001) Initiation factors in the early events of mRNA translation in bacteria. Cold Spring Harbor Symp Quant Biol 66:363–376

Gümüşel F, Kiremit N, Issever S, Ogras TT, Bermek E (1996) PCR-cloning of the *pac* gene from *E. coli* ATCC 11105-Observations on the expression of a recombinant periplasmic protein. Biochem Mol Biol Int 38:315-324

Gümüşel F, Ozturk SI, Korkut NK, Gelegen C, Bermek E (2001) Cloning and expression of the penicillin acylase gene (*pac*) from *E. coli* ATTCC 11105. Enzyme Microbl Tech 29:499-505

Hewitt L, Kasche V, Lummer K, Lewis RJ, Murshudov GN, Verma CS, Dodson GG, Wilson KS (2000) Structure of a slow processing precursor penicillin acylase from

Escherichia coli reveals the linker peptide blocking the active-site cleft. J Mol Biol 302: 887-898

Hinsley AO, Stanley NR, Palmer T, Berks BC (2001) A naturally occuring bacterial Tat signal peptide lacking one of the 'invariant' arginine residues of the consensus motif. FEBS Lett 497:45-49

Hwang LHS, Chen BF, Lin HM, Soong LL, Jou YS, Chang WC, Liu ST (1990) The striking effects of spacer and terminator sequences on the synthesis of porcine growth hormone in *Escherichia coli.* Biotechnol Lett 12:335-340

Ignatova Z, Hörnle C, Nurk A, Kasche V (2002) Unusual signal peptide directs penicillin acylase from *Escherichia coli* to the Tat translocation machinery. Biochem Biophys Res Commun 291:146–149

Issever Ozturk S (2002) The Investigation of the effects of the modifications introduced by Site-Directed Mutagenesis on the gene control region in the penicillin acylase (PA) model system and determination of the promotor from which gene expression directed. Dissertation, Istanbul University, TURKEY

Keilmann C, Wanner G, Böck A (1993) Molecular Basis of the exclusive low-temperature synthesis of an enzyme in *E. coli*: Penicillin Acylase. Biol Chem Hoppe-Seyler 374: 983-992

Krzeslak J, Braun P, Voulhoux R, Cool RH, Quax WJ (2009) Heterologous production of *Escherichia coli* penicillin G acylase in *Pseudomonas aeruginosa*. J Biotechnol 142:250-8

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-5

Laursen BS, Steffensen S, Hedegaard J, Moreno JM, Mortensen KK, Sperling-Petersen HU (2002) Structural requirements of the mRNA for intracistronic translation initiation of the enterobacterial *infB* gene. Genes Cells 7:901-10

Looman AC, Bodlaender J, Comstock LJ, Eaton D, Jhurani P, de Boer HA, van Knippenberg PH (1987) Influence of the codon following the AUG initiation codon on the expression of a modified *lacZ* gene in *Escherichia coli*. EMBO J 6:2489-2492

Ma J, Campbell A, Karlin S (2002) Correlations between Shine-Dalgarno sequences and gene features such as predicted expression levels and operon structures. J Bacteriol 184:5733-5745

Morales V, M Backman A, Bagdasarian M (1991) A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene 97:39–47

Natale P, Bruser T, Driessen AJ (2008) Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane-distinct translocases and mechanisms. Biochim Biophys Acta 1778:1735-1756

Shine J, L Dalgarno (1974) The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc Natl Acad Sci USA 71:1342-1346

Stenström CM, Holmgren E, Isaksson LA (2001a) Cooperative effects by the initiation codon and its flanking regions on translation initiation. Gene 273: 259-265

Stenström CM, Jin H, Major LL, Tate WP, Isaksson LA (2001b) Codon bias at the 3'-side of the initiation codon is correlated with translation initiation efficiency in *Escherichia coli*. Gene 263:273–284

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure, and some applications. Proc Natl Acad Sci USA 76:4350-4

Wexler M, Sargent F, Jack RL, Stanley NR, Bogsch EG, Robinson C, Berks BC, Palmer, T (2000) TatD is a cytoplasmic protein with DNase activity. No requirement for TatD family proteins in Sec independent protein export. J Biol Chem 275:16717–16722

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors, and host strains: Nucleotide sequences of the M13 mp18, and pUC19 vectors, Gene 33:103-19

Zalucki YM, Beacham IR, Jennings MP (2009) Biased codon usage in signal peptides: a role in protein export. Trends Microbiol 17:146-50

Figure captions

Fig. 1 PA activity in the total cell fractions of *E. coli* strains containing pUC19 constructs. Strains were grown for 20 hours at 26ºC, after which activity was determined (data represent the average of three experiments)

Fig. 2 PA activity in the total cell fractions of strains containing pMMB constructs, in *E. coli* MC4100 (dark shaded bars) and *E. coli* DADE (light shaded bars). Strains were grown for 20 hours at 26ºC, after which activity was determined (data represent the average of three experiments)

Fig. 3 Western blot analysis of total cell lysate from wild type *E. coli* MC4100 and *E. coli* DADE containing pMMB constructs with pac_{wt}, Mut6 or Mut7 (grown for 20 h at 26ºC with 0.1 mM of IPTG). An equivalent number of cells was loaded in each lane. The primary antibody used was a polyclonal antibody against PA. *lane 1*, *E. coli* MC4100 (pMMB-PACwt); *lane 2*, *E. coli* DADE (pMMB-PACwt); *lane 3*, purified PA; *lane 4*, *E. coli* MC4100 (pMMB-PACOA7); *lane 5*, *E. coli* DADE (pMMB-PACOA7) *lane 6*, *E. coli* MC4100 (pMMB-PACOA6); *lane 7*, E. *coli* DADE (pMMB-PACOA6)

Tables

Table 1 Primers used in this study

List of oligonucleotides	
Primer	Oligonucleotide sequence
PA6	5'-TACGCCTGCCAGAGGTAATTATATGAAAAATAGAAATCG -3'
PA7	5'-TACGCCTGCCAGAGGAAAAATTATATGAAAAATAGAAATCG-3'
PA8	5'-TACGCCTGCCAGAGGAAATTATATGAGAAATAGAAATCG-3'
PA9	5'-TACGCCTGCCAGAGGAAATTATATGAAAAATAAAAATCG-3'
PA10	5'-TACGCCATGGTGCCCACAAATATCAT-3'
PA11	5'-TGCCGTCTAGACGTTGCTAGTATCAATTCG-3'
PA12	5'-TCCGGGGTACCTTATCTCTGAACGTGCAACAC-3'
PA14	5'-TACGTGGTACCTTAAGCCCGAAAGCCCTCA-3'
PA16	5'-TACGTGAATTCCGGCGAAGTCTCCGTTG-3'

Table 2 Nucleotide sequences of Shine-Dalgarno, spacer region and downstream region of the wt and mutant *pac* genes

SD	Spacer region	Coding region	Mutations	Source
SD2 -10.....GAGGA SD1	TACA 4nt	ATG AAA AAT AGA AAT….. Met Lys Asn Arg Asn	→pac_{wt}	Gumusel et al. 2001
SD2 -10…..GAGGA SD1	AATTAT 6nt	ATG AAA AAT AGA AAT….. Met Lys Asn Arg Asn	→S_{pac}	Issever Ozturk 2002
-10……GAGG SD1	TAATTAT 7 nt	ATG AAA AAT AGA AAT….. Met Lys Asn Arg Asn	→Mut4	This study
SD2 -10.....GAGGA SD1	AAAATTAT 8 nt	ATG AAA AAT AGA AAT….. Met Lys Asn Arg Asn	→Mut5	This study
SD2 -10.....GAGGA SD1	AATTAT 6 nt	ATG **AGA** AAT AGA AAT….. Met Arg Asn Arg Asn	→Mut6	This study
SD2 -10.....GAGGA SD1	AATTAT 6 nt	ATG AAA AAT **AAA** AAT….. Met Lys Asn Lys Asn	→Mut7	This study

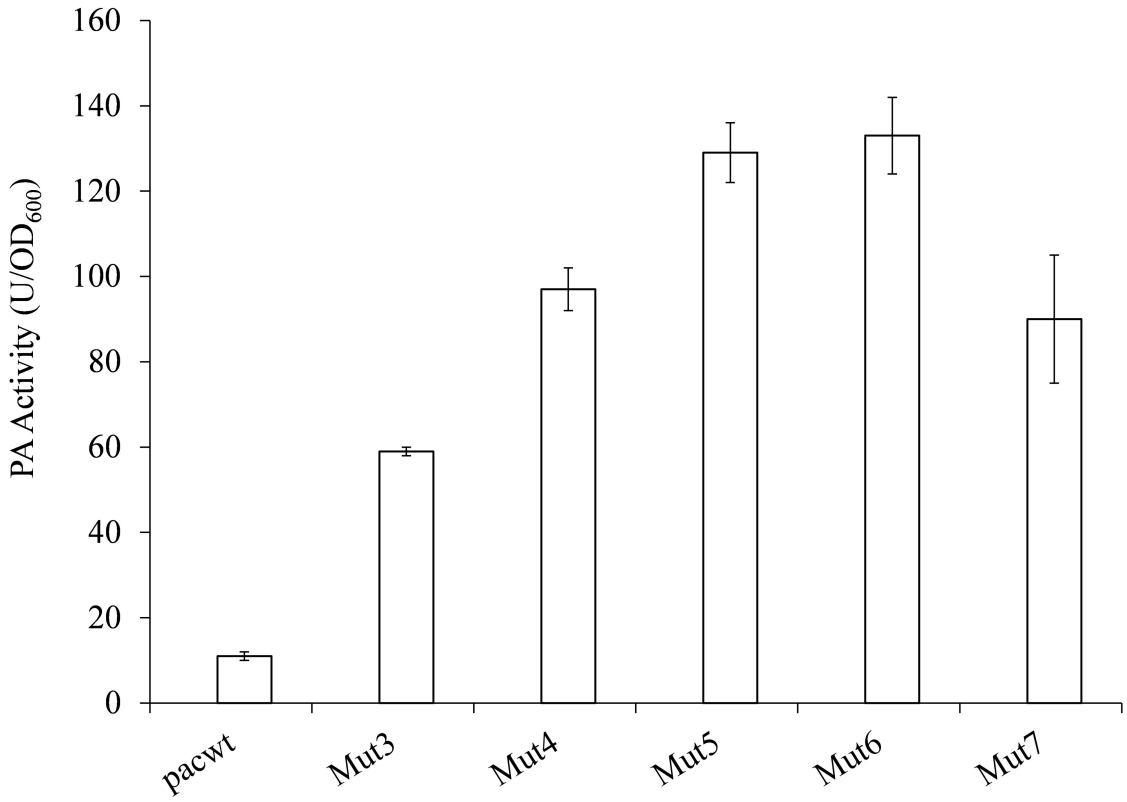
160
140
120
100
80
60
40
20
0
PA Activity (U/OD600)
pacwt
Mut3
Mut4
Mut5
Mut6
Mut7

Fig 1

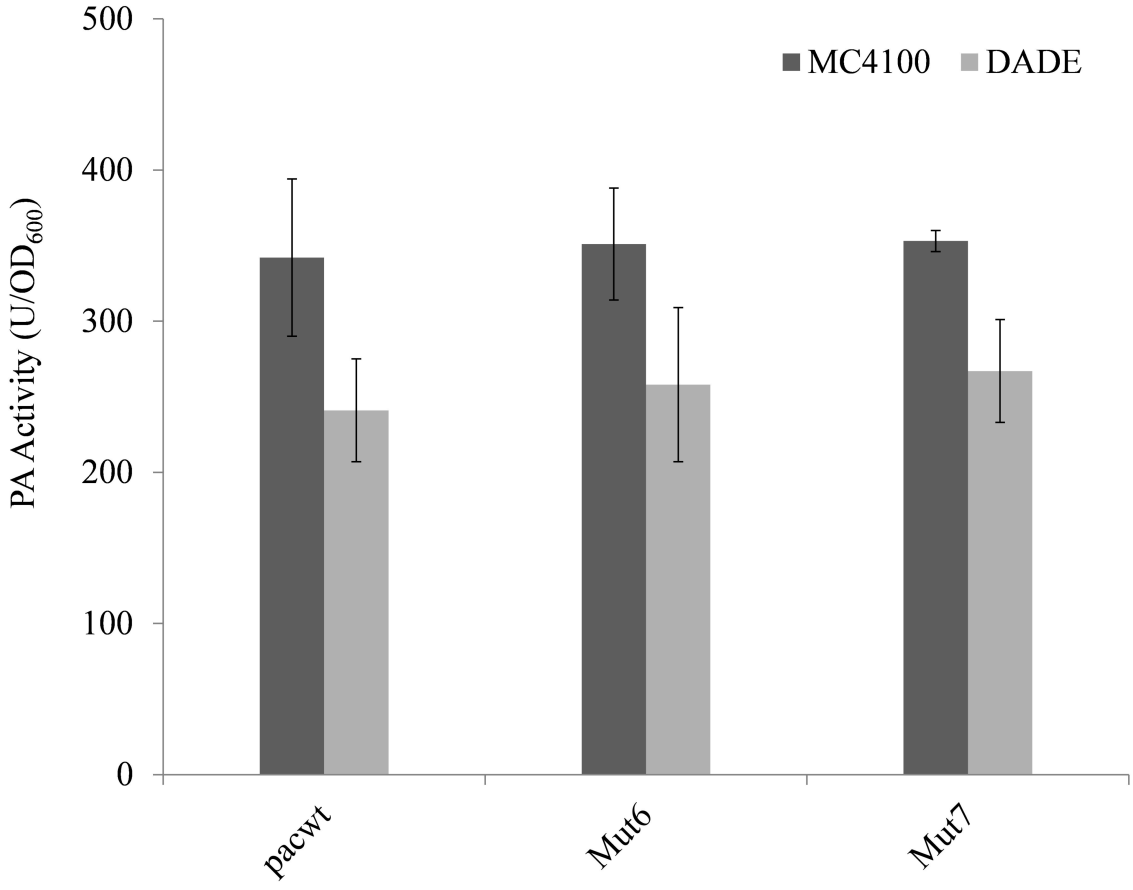
500
400
300
200
100
0
PA Activity (U/OD600)
MC4100
DADE
pacwt
Mut6
Mut7

Fig 2

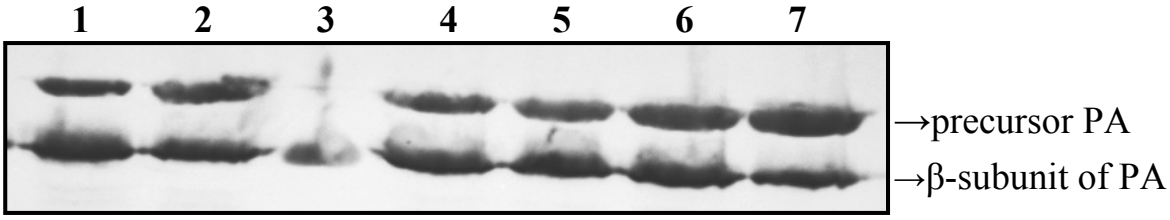

Fig. 3